**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/28, 15/56 — A1

(11) International Publication Number: **WO 98/45417**

(43) International Publication Date: 15 October 1998 (15.10.98)

(21) International Application Number: PCT/US98/07192

(22) International Filing Date: 8 April 1998 (08.04.98)

(30) Priority Data:
60/043,119 9 April 1997 (09.04.97) US

(71) Applicant *(for all designated States except US)*: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ZEIKUS, Joseph, G. [US/US]; 2529 Dustin, Okemos, MI 48864 (US). VIEILLE, Claire [US/US]; 2715 Fernwood, Lansing, MI 48912 (US). SAVCHENKO, Alexei [US/US]; 1312i University Village, East Lansing, MI 48023 (US). DONG, Guoqing [CN/CA]; 195 DaFoe Road, Winnipeg, Manitoba R3T 2M9 (CA).

(74) Agent: BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202–4497 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: HYPERTHERMOSTABLE α–AMYLASE

(57) Abstract

A preparation of Pyrococcus furiosus extracellular α–amylase is disclosed. In one embodiment, this α–amylase has an optimal temperature of 100 °C, a pH optimum of 5.5–6.0, and a half–life at 98 °C of between 12 and 14 hours. In another embodiment, the present invention is a gene construct encoding an extracellular α–amylase with an optimum temperature of 100 °C.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# HYPERTHERMOSTABLE α-AMYLASE

## TECHNICAL FIELD

In general, the field of the present invention is starch-hydrolyzing enzymes. Specifically, the field of the present invention is α-amylase enzymes.

## BACKGROUND ART

α-Amylases (EC 3. 2. 1. 1) are endo-acting enzymes that hydrolyze starch by cleaving α-1,4-glucosidic linkages at random. They are among the most important commercial enzymes having wide applications in starch processing, brewing and alcohol production, textile, and other industries. Numerous α-amylases have been characterized and their genes cloned from eubacteria, fungi, plants, and animals. With the exception of one eubacterial and one archaeal enzymes (18), they all belong to a same α-Amylase family, sharing a similar structure, similar catalytic site, and same catalytic mechanism (22).

α-Amylases contain three domains: (i) domain A corresponds to an $(\alpha_1/\beta)_8$ barrel; (ii) in this barrel, the $\beta_3 \rightarrow \alpha_3$ loop is very long and represents a second domain, domain B; Domain C is a separate globular domain composed of β-strands arranged in a Greek key motif (9). Four highly conserved regions come together through the interaction of domains A and B to form the active center, substrate binding site, and a $Ca^{2+}$ binding site. The $Ca^{2+}$ cation is essential for enzyme folding (9), optimal activity, and stability (43).

Since starch starts being soluble only at 100°C and above, the majority of α-amylase industrial applications require their use at temperatures up to 110°C (17). The most thermostable α-amylase (TAKA-THERM α-amylase) used in industry was purified from *Bacillus licheniformis*. It has an optimal temperature of 90°C and requires additional $Ca^{2+}$ for its thermostability (43).

Hyperthermophilic archaea are attracting increasing applied research attention since their enzymes show extreme thermostability (2; 42). Many hyperthermophiles can grow on starch and other carbohydrates, suggesting that they express a variety of amylolytic enzymes that could be of industrial interest (1; 42).

Recently, several hyperthermostable amylolytic enzymes have been reported from *Pyrococcus furiosus* (7; 15; 23; 25), *P. woesei* (24) and *Thermococcus profundus* (11). Only the *P. furiosus* intracellular α-amylase gene was cloned and expressed in *E. coli* (26). Its sequence shared very low homology with other α-amylase sequences and did not display any of the four consensus regions.

Needed in the art of starch hydrolysis is an α-amylase with improved thermostable characteristics.

## DISCLOSURE OF THE INVENTION

In one embodiment, the present invention is an α-amylase enzyme with an optimal activity temperature of 100°C. Preferably, the enzyme has a pH optimal of 5.5-6.0 and a half-life at 98°C of 12-14 hours.

In another embodiment, the present invention is a preparation of *Pyroccus furiosus* extracellular α-amylase. In a preferred embodiment, the preparation has an optimal activity temperature of 100°C, an optimum pH of 5.5-6.0, and a half-life at 98°C of between 12 and 14 hours.

In one embodiment of the present invention, the amino acid sequence of the α-amylase comprises SEQ ID NO:1. In another embodiment, the amino acid sequence of the α-amylase comprises resides 27-460 of SEQ ID NO:1.

In another embodiment, the present invention is a gene sequence encoding the α-amylase described above. In one embodiment, the gene sequence comprises SEQ ID NO:2. In a more preferred embodiment, the gene sequence comprises residues 118-1497 of SEQ ID NO:2.

The present invention is also a method of hydrolyzing starch, comprising the step of mixing starch and the α-amylase described above under conditions suitable for enzyme activity, wherein starch is hydrolyzed into oligosaccharides, preferably $G_2$-$G_7$.

It is an advantage of the present invention that an enzyme is provided that is suitable for starch hydrolysis at temperatures greater than or equal to 100°C.

Other advantages, features and objects of the present invention will become apparent to one skilled in the art after evaluation of the specification, claims and drawings.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is the nucleotide sequence (SEQ ID NO:2) and deduced amino acid sequence (SEQ ID NO:1) of the *P. furiosus* extracellular α-amylase gene.

Fig. 2 is a sequence alignment of *P. furiosus* (Pfu) (SEQ ID NO:1) and *B. licheniformis* (Bli) (SEQ ID NO:3) extracellular α-amylases.

Fig. 3A, B, and C illustrate *P. furiosus* extracellular α-amylase behavior on 12% polyacrylamide native gel (A), SDS gel (B), and starch-containing SDS gel (C).

Fig. 4 demonstrates TAKA-THERM α-amylase behavior on (A) 12% SDS-PAGE and (B) starch-containing 12% SDS-PAGE.

Fig. 5 is a graph of the effect of temperature on the activities of *P. furiosus* extracellular α-amylase (●) and TAKA-THERM (■).

Fig. 6 illustrates the influence of pH on *P. furiosus* α-amylase (●) and TAKA-THERM (O) activities.

Fig. 7 is a graph of thermostabilities of the recombinant *P. furiosus* extracellular α-amylase (■,●) and TAKA-THERM (x, ❑) in the absence (■, ●, ❑) or presence (x) of 5 mM $Ca^{2+}$ at 90°C (x, ❑, ■) or 98°C (●).

## BEST MODES FOR CARRYING OUT THE INVENTION

The present invention is a preparation of α-amylase enzyme, wherein the enzyme has an optimal activity temperature of 100°C. Preferably, this enzyme is isolated from *Pyroccus furiosus* and, most preferably, has the amino acid sequence of SEQ ID NO:1.

The Examples below and Dong, et al. (Applied and Environmental Microbiology, 63[9]:3569-3576, September 1997, hereby incorporated by reference) describe the purification of the α-amylase of the present invention from *Pyroccus furiosus*. Applicants envision that the α-amylase of the present invention may be purified from other hyperthermostable organisms.

The present invention is also a method of producing the α-amylase described above. Most preferably, the method would involve using a DNA sequence encoding the enzyme, such as that described in SEQ ID NO:2, in a genetic construct to express the protein in a host organism.

The DNA sequence encoding the enzyme may also be created by genetic engineering methods known to one of skill in the art. For example, applicants have provided a sequence for the cloned gene from *Pyrococcus furiosus*, and one may use this sequence to create primers capable of amplifying the gene from *Pyrococcus furiosus* genomic DNA. Once one has recloned the gene from *Pyrococcus furiosus*, one may place this gene in an appropriate expression vector and express the α-amylase protein in an appropriate host organism.

One of skill in the art would realize that SEQ ID NO:2 is not the only nucleotide sequence that could encode an α-amylase of the present invention. One may make modifications, deletions and additions to this sequence and still encode a functionally equivalent protein. (By "functionally equivalent, we mean an α-amylase enzyme meeting the criteria defined below at (1),

(2) and (4).) For example, the region upstream of residue 118 and downstream of residue 1497 may be deleted because this region does not encode the α-amylase protein. Preferably, the DNA sequence of the present invention encodes the protein sequence of SEQ ID NO:1 or SEQ ID NO:1 with conservative functionally equivalent substitutions.

The enzyme of the present invention can be defined by the following characteristics:

(1) The enzyme of the present invention has an optimal temperature of between 95 and 105°C. Preferably, the optimal temperature is 100°C. The Examples below and Dong, et al. (supra) describe the determination of maximal enzymatic activity of an α-amylase by performing standard enzyme assays at different temperatures. One would examine a candidate enzyme in the same manner.

(2) The enzyme of the present invention has a half-life at 98°C of 12 to 14 hours, preferably 13 hours. The Examples below and Dong, et al. (supra) describe methods of determing half-life.

(3) Preferably, the enzyme of the present invention has an amino acid sequence of SEQ ID NO:1. However, Applicants envision that conservative or non-conservative substitutions and deletions or additions to SEQ ID NO:1 could result in a functionally equivalent enzyme.

(4) The enzyme of the present invention preferably has a pH optimal of 5.5-6.0. The Examples below and Dong, et al. (supra) describe techniques for determining optimal pH.

By "preparation" we mean an enzyme purification whereby the α-amylase is substantially purified from its host organism. A preparation will have a specific activity of at least 350 μ/mg at 98°C. Preferably, a purified enzyme would have a specific activity of at least ~3,500 U/mg at 98°C.

EXAMPLES

The Examples report the characterization of a hyperthermostable *P. furiosus* extracellular α-amylase. Its gene was cloned and expressed in *E. coli* and its sequence was determined. The recombinant enzyme was purified and characterized. Its catalytic and stability properties were compared to those of the commercial *B. licheniformis* α-amylase (TAKA-THERM).

1. Materials and Methods

*Bacterial strains and growth conditions*

*P. furiosus* DSM 3638 was cultivated as described previously (15) at 90°C∓2°C under anaerobic conditions. Cells were harvested in the stationary growth phase and stored at -20°C before use. *Escherichia coli* Sure strain (Stratagene, La Jolla, CA) was used as plasmid host and grown in LB medium at 37°C. Ampicillin (100 μg/ml) was added when required. Plasmid pUC18 (Pharmacia Piscataway, NJ) was used as cloning and sequencing vector.

*Library construction and screening*

*P. furiosus* chromosomal DNA was prepared as reported (3). The chromosomal DNA was partially digested with restriction enzyme *Sau*3A. Plasmid pUC18 cut with *Bam*HI and dephosphorylated was ligated with the 4-8 kb DNA fragments isolated by a 10-40% sucrose gradient. The ligation mixture was transformed into *E. coli* Sure by electroporation and the bacteria were plated on 1.5% agar LB ampicillin plates. After 16-20 hours incubation at 37°C, colonies were replicated onto a new set of LB ampicillin plates containing 1% phytagel instead of agar and 0.2% soluble starch. After overnight growth, the plates were incubated at 80°C for 8-10 hours. Amylase activity was detected by flooding the plates with $I_2$/KI.

*Nucleotide sequence determination*

Restriction analysis and plasmid DNA purification were performed as reported (3). Nested deletions for sequencing were generated on both sides of the insert according to Henikoff (20). Sequences were analyzed in both directions by the dideoxy chain termination technique (36) using Sequenase version 2.0 T7 DNA polymerase sequencing kit (U. S. Biochemicals, Cleveland, OH) and ThermoSequenase kit (Amersham Life Science, Arlington Heights, IL). Sequencing data were analyzed using the GCG Sequencing Analysis Software Package V 7.0 (13). The amino acid sequence of *P. furiosus* extracellular α-amylase was compared with other amylolytic enzymes available through the GenBank/EMBL Data Bank (IntelliGenetics Inc., Mountain View, CA). Two pairs of oligonucleotides (pair 1:5'-CAAATGTCACGTTGT ATGG-3', SEQ ID NO:4 and 5'-GAGAGTGGTGCAAAGGTC-3', SEQ ID NO:5 and pair 2: 5'-CTGGTGGTGACCTAGAATG-3', SEQ ID NO:6 and 5'-TATCTGTGTCATGATTGGC-3', SEQ ID NO:7) identical to different sequences of the pS4 insert were synthesized and used as primers for PCR with *P. furiosus* genomic DNA as template. The PCR products were analyzed on agarose gels and sequenced. Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility.

*Nucleotide sequence accession number*

The GenBank accession number for the sequence published in this paper is AF001268.

*Site-directed mutagenesis*

The initiation codon (GTG) of the *P. furiosus* extracellular α-amylase gene was changed to ATG using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with primers 5'-GAGGTGATCACATGAACATAAAG AAATIAACACC-3' (SEQ ID NO:8) and 5'-GGTGTTAATTTCTTTAT GTTCATGTGATCACCT-3' (SEQ ID NO:9).

*Enzyme purification*

All purification steps were performed at room temperature under aerobic conditions. When expressed in *E. coli*, the recombinant *P. furiosus* extracellular α-amylase was not secreted into the medium. Cells carrying the recombinant plasmid pS4 were grown in LB ampicillin. Cell homogenate was prepared by passing through a French press cell at 15,000 lb/inch$^2$. After heat treatment at 80°C for 15 min, the cell homogenate was centrifuged at 16,3000 x g for 20 minutes. The enzyme was precipitated by adding 60% $(NH_4)_2SO_4$ to the supernatant, and the pellet was resuspended in 50 mM sodium acetate buffer at pH 6.0.

The concentrated crude enzyme was loaded onto a Phenyl-Sepharose (Pharmacia Fine Chemica AB, Uppsala, Sweden) column (1.5 x 18 cm) equilibrated with 50 mM acetate buffer (pH 6.0). The column was washed with the same buffer, then with 50 mM Tris-HCl (pH 8.0). The enzyme was eluted with 6 M urea in 20 mM Tris-HCl (pH 9.4). After concentration in an ultrafiltration cell equipped with a 30,000 molecular weight cut-off membrane (Amicon, Beverly, MA) and dialysis against 50 mM Tris-HCl (pH 6.0), the enzyme was loaded onto a Phenyl-Sepharose column (1.5 x 18 cm) equilibrated with 50 mM Tris-HCl (pH 6.0). The column was washed with 50 mM Tris-HCl at pH 6.0, then at pH 8.0, and pH 9.4. Finally, the enzyme was eluted with 6 M urea in 20 mM Tris-HCl (pH 9.4). The fractions with α-amylase activity were pooled and concentrated by ultrafiltration (see above).

The concentrated enzyme was loaded onto a Sephacryl S200 (Pharmacia Fine Chemica AB, Uppsala, Sweden) column (1.5 x 80 cm) equilibrated with 20 mM Tris-HCl buffer (pH 9.4) containing 5% glycerol. The active fractions were concentrated by ultrafiltration (see above) and dialyzed against 50 mM sodium acetate buffer (pH 5.6) (buffer A). Commercial TAKA-THERM L-340 α-amylase (TAKA-THERM) from *B. licheniformis*, a gift from Genencor International Inc.

(Rochester, NY), was dialyzed against 50 mM sodium acetate buffer (pH 6.0).

The NH-terminus of the recombinant *P. furiosus* α-amylase was sequenced by the Michigan State University Macromolecular Facility.

*Enzyme assays*

*P. furiosus* extracellular α-amylase activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% soluble starch in buffer A at 98°C for 15 minutes. A control without enzyme was used. The amount of reducing sugar was measured by a modified dinitro salicylic acid method (5). One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose per minute under the assay conditions. TAKA-THERM was assayed at 90°C in 50 mM sodium acetate buffer (pH 6.0) containing 0.5 mM $Ca^{2+}$ (buffer B). Other conditions were the same as above. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

*Molecular mass determination*

A 0.5 x 45 cm column containing Sephacryl S200 was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. The purified sample and marker proteins (i.e., carbonic anhydrase [29,000], bovine serum albumin [66,000] alcohol dehydrogenase [150,000], and Blue Dextran [2,000,000] were applied to the column at the flow rate of 7 ml/h. Elutions of the marker proteins and the recombinant *P. furiosus* extracellular α-amylase were followed by 280 nm-UV detection and activity assay.

*Gel-electrophoresis*

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 12% polyacrylamide (27). Protein samples were

denatured by incubation with denaturing buffer (2% SDS and 0.64 M mercaptoethanol, final concentrations) at different temperatures in water or silicon oil baths for varying periods. Low molecular weight protein markers (Bio-Rad, Richmond, CA) were used as standards. Native polyacrylamide gel electrophoresis was performed in the same conditions as above except for the absence of SDS in the buffer system and in gel. Samples were heat treated in 0.1 M acetate buffer (pH 5.6) at different temperatures before loading. Proteins were stained by Coomassie Brilliant Blue R-250. For activity staining, 0.66% soluble starch was added during SDS-polyacrylamide gel preparation. After electrophoresis, starch-containing gels were washed with buffer A and incubated at 90°C for 10 minutes in buffer A. Enzyme activity was visualized by flooding with a $I_2$/KI solution. Gels containing TAKA-THERM were washed with buffer B and incubated at 80°C for 10 minutes in buffer B. Other conditions were the same as above.

*Analysis of hydrolysis products*

The recombinant α-amylase (2.5 U/ml) was incubated at 90°C with 1% (wt/vol) soluble starch, pullulan, glycogen, amylose, amylopectin, or oligosaccharides. Samples were withdrawn after varying periods. Hydrolysis products were analyzed by high-performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) and CarboPac PAI column (4 mm x 250 mm) (Dionex system). Hydrolysis products were identified and quantified using the PEAK II computer software (SRI Instruments, Torrance, CA). Glucose ($G_1$), maltose ($G_2$), maltotriose ($G_3$), maltotetraose ($G_4$), maltopentaose ($G_5$), maltohexaose ($G_6$), and maltoheptaose ($G_7$) were the standards. Starch, pullulan, glycogen, amylose, amylopectin, and oligosaccharides were also incubated with TAKA-THERM (2.5 U/ml) at 80°C in buffer B, and hydrolysis products were analyzed for comparison.

*pH and temperature studies*

The optimal pH for *P. furiosus* extracellular α-amylase activity was determined at 98°C in 50 mM acetate buffer (pH 3.5-4.6) and 50 mM Tris-HCl buffer (pH 6-11). All pHs were adjusted at room temperature and corresponding pHs at high temperatures were calculated using $\Delta pKa/\Delta T°C$ = 0.000 and -0.031 for acetate and Tris, respectively (30). TAKA-THERM was assayed at 90°C in the same buffers in the presence of 0.5 mM $Ca^{2+}$.

The temperatures of maximal activity of *P. furiosus* α-amylase and TAKA-THERM were determined by performing standard enzyme assays at different temperatures.

For stability studies at high temperatures, both enzymes were EDTA-treated. They were first dialyzed extensively against buffer A (*P. furiosus* α-amylase) or buffer B without $Ca^{2+}$ (TAKA-THERM) containing 2 mM EDTA, then twice against the same buffers without EDTA. Enzyme thermal inactivation studies were performed by incubating 1 ml Gas Chromatography tubes (Alltech Associates, Deerfield, IL) that contained 800 µl purified enzyme in 0.1 M acetate buffer (pH 5.6 for the *P. furiosus* α-amylase and pH 6.0 for TAKA-THERM) in the presence or absence of 5 mM $Ca^{2+}$ at 90°C or 98°C. After various incubation periods, samples were withdrawn and tested for residual α-amylase activity under each enzyme's standard assay conditions.

*α-amylase overexpression in E. coli*

The *P. furiosus* α-amylase gene was amplified by PCR without its signal peptide. Oligonucleotide 5'-AGCTAGCTTGGAGCTTGAAGAGGGAG-3' (SEQ ID NO:10) was used as forward primer. Sequence AAATCA encoding the two N-terminal residues Lys-Tyr was substituted by GCTAGC, encoding Ala-Ser and creating an *Nhe*I site. Oligonucleotide 5'-ACTCGAGACCACAATAACTCCATACGGAG-3' (SEQ ID NO:11) was used as reverse primer. Sequence GITGGG (SEQ ID NO:12) encoding the C-terminal residues Val-Gly

was substituted by CTCGAG (SEQ ID NO:13), encoding Leu-Glu and creating a *Xho*I site. The amplified gene was cloned in pCR2.1 (Invitrogen, Carlsbad, CA) and its sequence was verified. It was then subcloned in pET21 (Novagen, Madison, WI), yielding recombinant plasmid pET213. In this construct, *P. furiosus* α-amylase is expressed with a $(His)_6$ C-terminal tail that allows its purification by Ni-NTA resin affinity chromatography. Recombinant plasmid pET213 was transformed in *E. coli* BL21(DE3) (Novagen, Madison, WI). BL21(DE3)(pE1213) was grown in LB medium to the end of the exponential phase and T7 RNA polymerase-dependent expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (6 mM final concentration). After three hours induction, cells extracts were prepared and analyzed by SDS-PAGE and activity assay.

2. Results

*Cloning and sequencing of the gene encoding P. furiosus extracellular α-amylase*

Among about 10,000 clones screened on starch-containing plates, two colonies developed a clear halo. Both transformants expressed thermostable α-amylase activity but no pullulanase activity. They both constitutively expressed the α-amylase in a starch-free medium in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG), the inducer of the lac promoter. The two recombinant plasmids were shown by restriction analysis to contain overlapping inserts. The smallest, pS4, carried a 2.7 kb insert, and was selected for further studies. Plasmid pS4's insert was sequenced entirely. To confirm that the insert corresponded to *P. furiosus* genomic DNA, two pairs of oligonucleotides corresponding to different pS4 sequences were used as primers in PCR reactions. With *P. furiosus* genomic DNA as template, the sequences of the PCR products were identical to the corresponding sequences in the pS4

insert, indicating that the insert did, indeed, come from *P. furiosus* chromosomal DNA. The pS4 insert contained a single complete open reading frame (ORF1) (Fig. 1).

Fig. 1 is the nucleotide sequence and deduced amino acid sequence of the *P. furiosus* extracellular α-amylase gene. Referring to Fig. 1, the NH-terminus of the recombinant protein is underlined with asterisks. The signal peptide is in italics. Putative promoter sequence, ribosome binding site (RBS), initiation codon, and transcription termination sequence are in bold. The -35 and -10 regions potentially recognized as *E. coli* promoters are underlined. The four conserved regions are framed and numbered.

The 491-residues polypeptide encoded by ORF1 showed an overall 35.7% identity to *B. licheniformis* α-amylase (Genbank accession no. m38570), indicating that ORF1 encoded the α-amylase expressed by pS4. The N-terminal sequence of the *P. furiosus* recombinant α-amylase was determined. It was identical to residues KYLEL located 58-62 residues downstream of ORF1's first ATG (Fig. 1).

Since most α-amylases, including the *B. licheniformis* enzyme, are extracellular enzymes, we checked if ORF1's 57 first residues could correspond to a signal peptide. The 26-residues stretch located just upstream of the KYLEL sequence (Fig. 1) showed all the characteristics of a prokaryotic signal peptide (Watson, 1984). It showed 66.7% and 57.7% similarity to *P. furiosus* (15) amylopullulanase and pyrolysin (46) signal peptides. This 26-residues sequence starts with a valine encoded by GTG (Fig. 1).

At least two *Pyrococcus* genes, the genes for *P. furiosus* intracellular α-amylase (26) and *P. woesei* glyceraldehyde-3 phosphate dehydrogenase (48), have been shown to have a GTG start codon. Several arguments tend to confirm that this new *P. furiosus* α-amylase gene also starts with GTG. (i) The similarity with *B. licheniformis* α-amylase clearly starts at the level of

this residue, at the N-terminus of the *B. licheniformis* α-amylase signal peptide (Fig. 2). (ii) The sequence GGAGGT located 5 nt upstream of the "starting GTG" (Fig. 1) represents a putative ribosome binding site (RBS). This sequence is identical to the *P. furiosus* maltose-regulated (34) *mlrA* and pyrolysin (46) genes's RBS's. No putative RBS could be identified upstream of any of the two ATG's preceding the "starting GTG". (iii) 52 nt upstream of the "starting GTG" (but downstream of the two ATG's) the sequence TTTATA (Fig. 1) is identical to the consensus defined as box A in archaeal promoters (19). (iv) Since GTG is rarely used as starting codon in *E. coli* genes, the "starting GTG" was mutagenized into ATG. Expression of the mutant enzyme in *E. coli* increased eight times compared to the wild-type enzyme (not shown). All these evidences showed that ORF1 encoded a *P. furiosus* extracellular α-amylase containing a 26-residues signal peptide. ORF1 stop codon was immediately followed by a 19-residues stretch of pyrimidines containing the sequence TTTTTCT typical of archaeal transcription termination signals (33). Two truncated ORF's (not shown but in Genbank) were detected upstream and downstream of ORF1, in the opposite orientation. Neither of them showed significant homology to any sequence present in the GenEMBL database.

The G+C content of this new *P. furiosus* α-amylase gene was 41.9%, slightly higher than the value (38%) reported for the total genome (16). As has been seen in other genes sequenced from hyperthermophiles, A and T were the preferred bases (62%) in the third position of codons (48). Proline and threonine codons ending with G were rarely used. Like other reported hyperthermophilic archaeal protein genes (39), AGG and AGA arginine codons were strongly preferred. Interestingly, *P. furiosus* extracellular α-amylase contained five cysteines.

Archaeal genes can generally not be directly expressed from their own promoters in *E. coli*. The two

sequences TTCACA N17 TTATAT and TTTATA N17 TACATT located 80-52 and 58-29 nt upstream of the GTG start codon, respectively, are close to the *E. coli* consensus promoter sequence. One of them is probably responsible for *P. furiosus* α-amylase gene expression in *E. coli*.

*Comparison of P. furiosus and B. licheniformis α-amylase sequences*

*P. furiosus* extracellular α-amylase showed 45-56% similarity and 20-35% identity to eubacterial α-amylases and other enzymes of the α-amylase family (e.g., neopullulanase, pullulanase, isoamylase, amylopullulanase) (not shown). The closest enzyme was *B. licheniformis* α-amylase (55.7% similarity and 35.7% identity) (Fig. 2).

Fig. 2 is a sequence alignment of *P. furiosus* (Pfu) (SEQ ID NO:1) and *B. licheniformis* (Bli) (SEQ ID NO:3) extracellular α-amylases. Vertical lines and columns denote identical and similar residues, respectively. Numbering starts after the signal peptides. Signal peptides are in italics. The four active site conserved regions are in bold. (α/β) Barrel (domain A) and domain B are framed and shadowed, respectively. Underlined sequences correspond to the secondary structures in the *B. licheniformis* α-amylase (α/β) barrel, as described by Machius, et al. (28). Corresponding secondary structures are indicated under the sequence. *B. licheniformis* α-amylase residues involved in $Ca^{2+}$ (*) and Cl (●) (28) are indicated under and above the sequence, respectively.

Conservation was not uniform along the whole sequence. In particular, the sequences corresponding to the (α/β) barrel domains (or domains A), domains B, and domains C showed 40%, 22%, and 30% identity, respectively. *P. furiosus* α-amylase is 10% shorter than the *B. licheniformis* enzyme. *P. furiosus* α-amylase domains A, B, and C were 9%, 29%, and 17% shorter, respectively, than the corresponding domains in the *B. licheniformis* enzyme. In the best conserved domain, the

(α/β) barrel, most secondary structures were conserved, with the exception of helices $\alpha_3$ and $\alpha_4$ which were significantly shorter in the *Pyrococcus* enzyme (Fig. 2).

Another striking difference in the (α/β) barrel was the presence of two nine-residues deletions in loops $B_7 \rightarrow \alpha_7$ and $B_8 \rightarrow \alpha_8$ of the *Pyrococcus* enzyme (Fig. 2). The four active site consensus regions characteristic of the α-amylase family were present in the *P. furiosus* α-amylase (Fig. 2). From the three *B. licheniformis* α-amylase residues (Asn104, Asp200, and His235) involved in $Ca_2$ binding (28), only Asn104 was conserved in the *Pyrococcus* enzyme (Fig. 2). Interestingly, the two strictly conserved *B. licheniformis* α-amylase residues involved in chloride binding (Arg229 and Asn326) are present in the *Pyrococcus* enzyme (Fig. 2).

The amino acid compositions of the two enzymes (not shown) differed in two ways. (i) The *P. furiosus* enzyme was more negatively charged (net charge of -21) than the *B. licheniformis* enzyme (net charge of -8). This difference is mainly due to a lower number of Lys plus Arg residues in the *P. furiosus* enzyme (35 compared to 54 in the *B. licheniformis* enzyme), and is probably responsible for the two-units difference between the enzymes's isoelectric points (pIs of 4.78 and 6.83 for *P. furiosus* and *B. licheniformis* enzymes, respectively). (ii) The *P. furiosus* enzyme contained 5% more aromatic residues (18.5% against 13.7%) than the *B. licheniformis* enzyme.

*P. furiosus* extracellular α-amylase was also compared to the two other *P. furiosus* amylolytic enzymes sequenced so far, the *P. furiosus* intracellular α-amylase (26) and amylopullulanase (15). No significant similarity was found with either of these enzymes.

*Purification of the P. furiosus extracellular α-amylase*

The *P. furiosus* extracellular α-amylase was very thermostable. It did not lose any activity when the *E.*

*coli* cell homogenate was treated at 100°C for 20 minutes. However, more than 50% of its activity was lost after centrifugation due to coprecipitation of the enzyme with cell debris and other denatured proteins. The precipitated enzyme remained active and was detected after resuspending the precipitate. Triton X-100 did not significantly prevent the protein from coprecipitating. To reduce coprecipitation, the cell homogenate was heated to 80°C for 15 minutes.

The α-amylase was so hydrophobic that it was directly absorbed onto the Phenyl-Sepharose column in the absence of any salt. The hydrophobic interaction was weakened by raising the buffer pH. The enzyme was totally eluted by 6 M urea at pH 9.4. The purified α-amylase displayed one protein band on native polyacrylamide gel (Fig. 3A) and had a specific activity of 3900 U/mg at 98°C.

*Properties of the P. furiosus extracellular α-amylase vs TAKA-THERM*

The approximate molecular weight of the recombinant *P. furiosus* extracellular α-amylase was 100,000 as estimated by gel filtration. This value was just twice the molecular weight calculated according to its deduced polypeptide sequence, indicating that the protein was a homodimer.

Experiments were initiated to test whether the α-amylase was active as a dimer or a monomer. Fig. 3 illustrates *P. furiosus* extracellular α-amylase behavior on 12% polyacrylamide native gel (A), SDS gel (B), and starch-containing SDS gel (C). Referring to Fig. 3, A: Protein sample was not denatured before loading, and B and C: Protein samples were treated in denaturing buffer at 60°C (lanes 1), 90°C (lane 2), 100°C (lane 3), or 110°C (lanes 4) for 10 minutes before loading. Gels A and B were stained by coomassie blue. Gel C was stained for α-amylase activity.

Native gel showed one protein band (Fig. 3A). On SDS-PAGE, when denaturing temperatures were under 60°C the protein remained dimeric with an apparent molecular weight (MW) of 66,000. If denaturation was performed at 90°C or above, a 44,000 MW protein band appeared. This MW was lower than the 52,000 MW expected from the sequence. At 110°C, all the dimeric enzyme had dissociated into monomers of 44,000 MW along with protein degradation products. Both the dimer and monomer showed α-amylase activity (Fig. 3C). Due to the affinity of the dimeric enzyme for starch, the dimer migrated slower on starch-containing SDS gel than on SDS gel in the absence of starch. A low molecular weight protein band appeared at the bottom of SDS gels from protein degradation. Protein degradation in SDS buffers was also observed with other proteins (25).

TAKA-THERM was more sensitive to denaturation than the *P. furiosus*. During denaturation at or below 60°C, the protein retained its dimeric form with an apparent molecular weight of 122,000. Denaturing the enzyme at 90°C led to complete dissociation into monomers that migrated at 59,000 (Fig. 4A). TAKA-THERM only displayed α-amylase activity as a dimer (Fig. 4B).

Fig. 4 demonstrates TAKA-THERM α-amylase behavior on (A) 12% SDS-PAGE and (B) starch-containing 12% SDS-PAGE. Protein samples were treated in denaturing buffer at 60°C (lanes 1), or 90°C (lanes 2) for 10 minutes before loading. Gel A was stained by coomassie blue and gel B was stained for α-amylase activity.

Fig. 5 demonstrates the effect of temperature on the activities of *P. furiosus* extracellular α-amylase (●) and TAKA-THERM (■). TAKA-THERM was assayed in the presence of 0.5 mM $Ca^{2+}$. *P. furiosus* α-amylase displayed no activity at room temperature.

Referring to Fig. 5, α-amylase activity increased with temperature up to an optimum at 100°C. TAKA-THERM showed about 22% activity at room temperature and reached

its highest activity at 90°C. Both Arrhenius plots were linear (Fig. 5, inset). Activation energies were 70 kJ mole$^{1}$ and 17 kJ mole$^{1}$ for the *P. furiosus* α-amylase and TAKA-THERM, respectively, as calculated from the Arrhenius equation: $Ln(k) = B - E_{act}/RT$ (where k = rate constant; B = constant; $E_{act}$ = activation energy; R = molar gas constant [8.314 J mol$^{1}$ K$^{1}$]; and T = absolute temperature). Unlike TAKA-THERM's activity which increased by approximately 10% in the presence of 0.5 mM $Ca^{2+}$, the *Pyrococcus* enzyme did not require $Ca^{2+}$ for activity.

The *P. furiosus* extracellular α-amylase had a lower optimal pH than TAKA-THERM (Fig. 6). Fig. 6 illustrates the influence of pH on *P. furiosus* α-amylase (●) and TAKA-THERM (O) activities. *P. furiosus* α-amylase was assayed at 98°C without $Ca^{2+}$, whereas TAKA-THERM was assayed at 90°C in the presence of 0.5 mM $Ca^{2+}$.

The *Pyrococcus* enzyme showed 80% activity or more between pHs 4.5-7.0, with an optimal pH around 5.5-6.0. TAKA-THERM was optimally active around pH 7.0-8.0.

Fig. 7 compares the thermostabilities of the recombinant *P. furiosus* extracellular α-amylase (■,●) and *TAKA-THERM* (x, ❑) in the absence (■, ●, ❑) or presence (x) of 5 mM $Ca^{2+}$ at 90°C (x, ❑, ■) or 98°C (●).

The *Pyrococcus* enzyme's inactivation time courses in the presence and absence of $Ca^{2+}$ (not shown) were identical at the two temperatures tested (90 and 98°C). $Ca^{2+}$, however, strongly stabilized TAKA-THERM. At 90°C, its half-life increased more than 20-fold in the presence of 5 mM $Ca^{2+}$. One hour incubation at 98°C completely inactivated TAKA-THERM, even in the presence of 5 mM $Ca^{2+}$.

*Substrate specificity and hydrolysis product analysis*

The *P. furiosus* extracellular α-amylase hydrolyzed a wide variety of substrates such as soluble starch, amylose, amylopectin, glycogen and oligosaccharides. The enzyme did not hydrolyze pullulan, cyclodextrins,

sucrose, and maltose. α-Amylases can be classified into liquefying-type and saccharifying-type enzymes. Liquefying α-amylases have much wider commercial applications. Table 2 compares the hydrolysis products of *P. furiosus* and TAKA-THERM α-amylases. Like Taka-therm, the *P. furiosus* extracellular α-amylase was a liquefying enzyme. The main products of polysaccharide hydrolysis were $G_2$-$G_7$. A low amount of glucose was formed after long hydrolysis periods. The *P. furiosus* extracellular α-amylase hydrolyzed long-chain oligosaccharides faster than shorter chain oligosaccharides, as interpreted from the quantitation of products formed after short versus long incubation times (Table 3).

*α-amylase overexpression in E. coli*

Since very little α-amylase was produced from plasmid pS4 (about 1 mg/liter culture), we developed a construct that expressed more enzyme. In plasmid pET213, *P. furiosus* α-amylase gene was cloned under the control of the T7 promoter, without the sequence encoding its signal peptide. A band corresponding to a 45 kDa protein was observed on SDS-PAGE in crude extracts of BL21(DE3)(pET213) cultures after IPTG induction (not shown). This band was absent in crude extracts of uninduced BL21(DE3)(pET213) cultures. 38,000 U α-amylase activity at 98°C were obtained per liter of IPTG-induced BL21(DE3)(pET213), corresponding to the expression of 10 mg/liter α-amylase.

3. Discussion

With the cloning and characterization of the *P. furiosus* extracellular α-amylase described herein, three *P. furiosus* amylolytic enzymes--intracellular α-amylase (25; 26), extracellular α-amylase, and amylopullulanase (15)--have now been characterized. The new α-amylase appeared extracellular and belonged to the main family of

α-amylases, as opposed to the α-amylase characterized by Laderman, et al. (25; 26) which was intracellular and did not show any similarity to the α-amylase family. The new enzyme did not display any pullulanase activity, and its sequence was not related to *P. furiosus* amylopullulanase.

Koch, et al. (23) described an extracellular α-amylase activity present in the supernatant of *P. furiosus* cultures. The activity they described corresponded to two starch-degrading protein bands of 96 and 136 kDa on native polyacrylamide gel. It is not clear from their work if these two bands correspond to one or two separate enzymes and if the 96 kDa band is similar to the enzyme described here. It is unlikely that these bands are the *P. furiosus* amylopullulanase (8; 15), since no pullulanase activity was detected in Koch, et al.'s enzyme preparation (23).

Another member of the order *Thermococcales*, optimally growing at 80°C, *Thermococcus profundus* produced two extracellular amylases, amylases S and L (11). With a molecular mass of 42 kDa on SDS-PAGE, amylase S is an α-amylase optimally active at pH 5.5-6.0 and 80°C, and does not require $Ca^{2+}$ for its activity. While no sequence is available for amylase S, its catalytic properties suggest that this enzyme is the counterpart in *T. profundus* of *P. furiosus* extracellular α-amylase. Amylase L, a bigger size enzyme, could correspond to the *P. furiosus* 136 kDa amylolytic enzyme detected by Koch, et al. (23).

We propose that the *P. furiosus* extracellular α-amylase and amylopullulanase are involved in starch degradation. A putative integral membrane protein encoded by an ORF located upstream of the amylopullulanase gene (15) might participate in transporting the starch hydrolysis products inside the cells where an α-glucosidase hydrolyses them to glucose (12). Because starch is typically an extracellular compound, the function of *P. furiosus* intracellular

α-amylase is not clear. This enzyme can degrade starch down to glucose and maltose plus a mixture of oligosaccharides, most of them $G_4$, $G_5$, and $G_6$. It is also able to synthesize $G_4$ and $G_6$ from maltose, and $G_4$, $G_5$, and $G_6$ from maltotriose (25). Its function might not be in starch catabolism. Several other starch-degrading hyperthermophilic eubacteria and archaea also contain two or more amylases. So far, though, all of them are extracellular (11; 18; 21; 23) or exposed on the cell surface (37).

The new *P. furiosus* α-amylase gene was preceded by a typical archaeal "TATA" box and a ribosome binding site. Generally, genes from hyperthermophilic archaea are not directly expressed in *E. coli*. Among all the genes reported from hyperthermophilic archaea, only *P. woesei* (35) and *P. furiosus* (15) amylopullulanase genes, *P. furiosus* β-glucosidase (45), and β-mannosidase (4) genes were expressed in *E. coli* from promoters present in their upstream non-coding sequences. Sequences reminiscent of -35 and -10 *E. coli* promoter sequences could be identified in front of most of these genes (see 15). One of the two sequences reminiscent of *E. coli* promoters and located upstream of the *P. furiosus* α-amylase gene probably allows the α-amylase to be expressed in *E coli*. This observation supports our earlier hypothesis (15) that direct expression of archaeal genes in *E. coli* requires a sequence reminiscent of an *E. coli* promoter.

The *P. furiosus* extracellular α-amylase is the first archaeal amylolytic enzyme described belonging to the α-amylase family. The characterization of extracellular α-amylases with similar properties from other hyperthermophilic archaea (11; 24) suggests that these enzymes also belong to the α-amylase family and that this enzyme family is widespread among the three kingdoms. A second α-amylase family composed so far of only two enzymes, the *Dictyoglomus thermophilum* AmyA α-amylase and the *P. furiosus* intracellular α-amylase, spreads already

in two kingdoms, eubacteria (*D. thermophilum*) and archaea (*P. furiosus*). It is not excluded that enzymes from this family be found in eukaryotes as well.

The *P. furiosus* α-amylase described here showed a high identity level to the *B. licheniformis* α-amylase. It contained the four consensus regions typical of the α-amylase family. With the exception of helices $\alpha_3$ and $\alpha_4$ which were shorter, the secondary structures of the α/β barrel domain were well conserved, in particular the β strands and the β→α loops which are all located in the direct vicinity of the active site (Fig. 2). The least conserved domain was domain B which was 30% shorter than in *B. licheniformis* enzyme, and showed low sequence conservation (Fig. 2).

Since the *P. furiosus* extracellular α-amylase was significantly more thermostable than the commercial TAKA-THERM enzyme from *B. licheniformis*, the two enzymes sequences were compared to look for potential stabilizing elements in the *Pyrococcus* enzyme. (i) The *Pyrococcus* enzyme was significantly more negatively charged (by 13 charge units) than the *B. licheniformis* enzyme. It is not clear how this difference can affect the enzyme stability. It might involve additional surface interactions with extracellular salts. This charge difference might affect the enzymes's optimum pH for activity and stability more than their thermostability, though. There is indeed a two-pH-units difference between the two enzymes's pIs and also almost a two-pH-units difference between their optimum pHs for activity (pH 5.5 and 7.5 for *P. furiosus* and *B. licheniformis* enzymes, respectively).

(ii) The *P. furiosus* enzyme contains 5% more aromatic residues (18.5% against 13.7%) than the *B. licheniformis* enzyme. Aromatic residues have been shown to form networks of potentially stabilizing aromatic interactions in some thermostable enzymes (38). The three-dimensional structure of the *Pyrococcus* enzyme is

probably required to study the extent to which the additional aromatic residues create new stabilizing interactions in this enzyme.

(iii) The *P. furiosus* enzyme is significantly shorter than its *B. licheniformis* counterpart. Increased enzyme compactness has often been suggested as a thermostabilizing factor (10). The variation in heat capacity ($\Delta C_p$) associated with protein unfolding is considered to be essentially related to the change in solvent accessible surface area (in particular the exposure of hydrophobic residues to water) (see 29). Because smaller native proteins have a smaller solvent-accessible surface area, their $\Delta C_p$ of unfolding are reduced, their stability curves are broader, and their melting temperatures are higher (see 29). The areas affected by deletions in the *Pyrococcus* enzyme are mostly regions with little secondary structure or higher flexibility (i.e., the regions most susceptible to unfold first) such as loops $\beta_7$-$\alpha_7$ and $\beta_8$-$\alpha_8$ in the ($\alpha/\beta$) barrel, or domain B, which is all together more susceptible to unfolding than the ($\alpha/\beta$) barrel.

(iv) Two of the $Ca^{2+}$ binding residues of *B. licheniformis* α-amylase (Asp200, and His235) are absent in the *Pyrococcus* enzyme. This absence is not surprising since the *Pyrococcus* enzyme does not require $Ca^{2+}$ for either its activity or its stability. In the α-amylases whose three-dimensional structure has been solved, $Ca^{2+}$ participates in stabilizing the interaction between the ($\alpha/\beta$) barrel and domain B by creating an ionic bridge between the two domains (9; 28). Since domain B is poorly conserved and significantly shorter in *P. furiosus* α-amylase, and since $Ca^{2+}$ is not required for the enzyme stability, a different type of interaction might exist between domains A and B in this enzyme, that does not involve a $Ca^{2+}$ cation.

(v) Unlike the *B. licheniformis* enzyme which does not contain any cysteine residue, the *P. furiosus* α-

amylase contains five cysteines, three in domain B and two in domain C. It is now well known that cysteine residues are among the residues most sensitive to degradation at high temperatures (44), and that they are usually rare in highly thermostable enzymes (48; 14) The presence of five cysteines in the *Pyrococcus* enzyme is, therefore, surprising Chung, et al. (11) reported that an accessible cysteine may be involved in *T. profundus* α-amylase catalysis. A similar situation can happen with the *Pyrococcus* enzyme if, as we think, the *Thermococcus* and *Pyrococcus* extracellular α-amylases are related.

Denaturation of the *P. furiosus* α-amylase dimer required harsh denaturing conditions. It required 2% SDS plus 0.64 M mercaptoethanol and temperatures above 90°C to dissociate into monomers. Unfolding was not complete, though, since the enzyme remained at least partially active. The apparently low MW of 44,000 (as compared to a 52,000 MW predicted from the sequence) observed for the monomer in these conditions might reflect the incomplete unfolding of the enzyme. Such a behavior was not observed with the *B. licheniformis* enzyme which, once it was dissociated into monomers, had an apparent 59,000 MW on SDS-PAGE identical to the 58,500 MW predicted from its sequence, and was completely inactive (Fig. 4).

Denaturation of the *P. furiosus* α-amylase dimer into the monomeric form occurred at temperatures twenty degrees higher than required for the TAKA-THERM enzyme. Notably, the monomer of *P. furiosus* α-amylase remained active, whereas the TAKA-THERM monomer was completely inactive. The apparent molecular weight of the *P. furiosus* extracellular α-Amylase dimer on SDS-PAGE was only 1.5 times higher than its monomer, indicating that the protein still retained its globular structure and moved faster on SDS-PAGE than expected from its actual molecular weight. This behavior was also observed with other proteins from hyperthermophiles (15; 35).

A *P. woesei* extracellular α-amylase has been purified and characterized by Koch, et al. (24). *P. furiosus* and *P. woesei* extracellular α-amylases are optimally active in the same conditions of pH and temperature and have similar resistance to thermal inactivation. Although the *P. woesei* enzyme was described as a 70,000 molecular weight enzyme--as indicated from migration on an SDS-PAGE--it could correspond to a dimeric enzyme showing an aberrant behavior in these electrophoresis conditions. Such a behavior was observed with the *P. furiosus* enzyme, when mild denaturation conditions were used. It migrated as a 66,000 molecular weight dimer, instead of a 52,000 monomer. *P. furiosus* and *P. woesei* extracellular α-Amylases seem to differ on two aspects. (i) The *P. woesei* enzyme shows almost six-times less specific activity than the *P. furiosus* enzyme (667 versus 3900 U $mg^{-1}$). (ii) Their amino acid compositions seem to be different. In particular, the *P. furiosus* enzyme contains half the threonine residues present in the *P. woesei* enzyme. *P. furiosus* and *P. woesei* amylopullulanases were shown to be significantly different in a few aspects (15). Although these two organisms are considered very close, they still contain quite different enzymes.

Table 2 summarizes the differences between extracellular α-Amylases from *P.furiosus* and *B. licheniformis* (TAKA-THERM). Starch liquefying requires using α-amylase at high temperatures (up to 110°C). TAKA-THERM has a wide application in industry today. *P. furiosus* extracellular α-amylase showed promising properties over TAKA-THERM: (1) The enzyme displayed higher optimal temperature and thermostability than TAKA-THERM. Therefore, starch liquefying can be performed at very high temperatures without a risk of losing activity; (2) The enzyme had a low optimal pH (pH 5.5 versus pH 7.5). Thus, starch liquefying and saccharifying can be

operated under more similar pH conditions. (3) Unlike TAKA-THERM, $Ca^{2+}$ was not needed for activity and thermostability. This could eliminate the ion-exchange step used to remove $Ca^{2+}$ which is an inhibitor during high fructose syrup production via glucose isomerase. (4) The *P. furiosus* enzyme is about twice as active as TAKA-THERM.

Table 1: Hydrolysis products of different substrates by the recombinant *P. furiosus* and Taka-therm α-amylases

| Substrate | Enzyme sources | Product (%)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $G_5$ | $G_6$ | $G_7$ | $>G_7$ |
| Soluble Starch | *P. furiosus* | 2.0 | 27.5 | 18.2 | 12.1 | 16.4 | 16.0 | 11.4 | 5.0 |
| | Taka-therm | 13.5 | 31.9 | 18.2 | 11.5 | 16.5 | 6.1 | 2.4 | 1.1 |
| Glycogen | *P. furiosus* | 1.0 | 15.7 | 14.9 | 14.7 | 15.4 | 15.1 | 7.8 | 15.3 |
| | Taka-therm | 9.9 | 26.4 | 13.7 | 12.6 | 14.6 | 5.8 | 4.9 | 12.9 |
| Amylose | *P. furiosus* | 2.4 | 41.7 | 23.2 | 11.4 | 11.9 | 9.5 | 0.0 | 0.0 |
| | Taka-therm | 15.4 | 37.5 | 22.1 | 10.3 | 14.6 | 0.0 | 0.0 | 0.0 |

*Incubation period was 46 hours.

Table 2: *P. furiosus* α-amylase product specificity: comparison of the hydrolysis products of different oligosaccharides.

| Substrate | Time Period (h) | Product/residual substrate (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $G_5$ | $G_6$ | $G_7$ |
| Maltotetraose | 0.83 | 0.0 | 2.3 | 13.0 | 84.8 | | | |
| | 46 | 0.2 | 25.2 | 24.7 | 49.5 | | | |
| Maltopentaose | 0.83 | 0.0 | 2.0 | 3.0 | 11.3 | 83.7 | | |
| | 46 | 0.6 | 19.2 | 24.4 | 21.1 | 35.0 | | |
| Maltohexaose | 0.83 | 0.1 | 1.5 | 1.8 | 2.0 | 14.2 | 80.4 | |
| | 46 | 1.6 | 18.3 | 18.2 | 14.5 | 19.2 | 28.1 | |
| Maltoheptoase | 0.83 | 1.2 | 4.2 | 0.9 | 1.7 | 9.1 | 15.6 | 67.4 |
| | 46 | 3.64 | 32.6 | 12.4 | 14.0 | 23.0 | 13.5 | 0.9 |

Table 3. Comparison of the general biochemical properties of the *P. furiosus* extracellular and *B. licheniformis* Taka-therm α-amylases

| Properties | *P. furiosus* | TAKA-THERM |
|---|---|---|
| Molecular weight | 100,000 | 122,000 |
| Specific activity (U/mg) | 3,900 | 2,000 |
| Subunit activity | Yes | No |
| Optimal pH | 5.5-6.0 | 7.0-8.0 |
| Optimal temperature | 100°C | 90°C |
| $Ca^{2+}$ requirement | No | Yes |
| Half-life at 98°C | 13 h | <1 hr |
| End product | $G_2$-$G_7$ | $G_1$-$G_6$ |

REFERENCES

1. Adams, M.W.W., "Enzymes and proteins from organisms that grow near and above 100°C," Ann. Rev. Microbiol. 47:627-658, 1993.

2. Adams, M.W.W., F.B. Perler, and R.M. Kelly, "Extremozymes: expanding the limits of biocatalysis," Bio/Technology 13:662-668, 1995.

3. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.), "Current Protocols in Molecular Biology," Greene Publishing & Wiley-Interscience, New York, 1993.

4. Bauer M.W., E.J. Bylina, R.V. Swanson, and R.M. Kelly, "Comparison of a β-glucosidase and a β-mannosidase from the hypertherrnophilic archaeon *Pyrococcus furiosus*: purification, characterization, gene cloning and sequence analysis," J. Biol. Chem. 271:23749-23755, 1996.

5. Bernfeld, P., "Amylases α- and β-," Methods Enzymol. 1:149-158, 1955.

6. Boel, E.L., Brady, A.M., Brzozowsk, Z., Derewenda, G.G., Dogson, V.J., Jensen, S.B., Petersen, H., Swift, L., Thim, and H.F. Woldike, "Calcium binding in α-amylases: An X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*," Biochemistry 29:6244-6249, 1990.

7. Brown, S.H., H.R. Costantino, and R.M. Kelly, "Characterization of amylolytic activities associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus*," Appl. Environ. Microbiol. 56:1985-1991, 1990.

8. Brown, S.H., and R.M. Kelly, "Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*," Appl. Environ. Microbiol. 59:2614-2621, 1993.

9. Buisson, G., E. Duee, R. Haser, and F. Payan, "Three dimensional structure of porcine pancreatic α-amylase at 2.9 Å resolution. Role of calcium in structure and activity," EMBO J. 6:3909-3916, 1987.

10. Chan, M.K., S. Mukund, A. Kletzin, M.W.W. Adams, and D.C Rees, "Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase," Science 267:1463-1469, 1995.

11. Chung, Y.C., T. Kobayashi, H. Kanai, T. Akiba, and T. Kudo, "Purification and Properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432," Appl. Environ. Microbiol. 61:1502-1506, 1995.

12. Costantino, H.R., S.H. Brown, and R.M. Kelly, "Purification and characterization of an α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115°C," J. Bacteriol. 172:3654-3660, 1990.

13. Devereux, J., P. Haeberli, and O. Smithies, "A comprehensive set of sequence analysis programs for the VAX." Nucleic Acids Res. 12:387-395, 1984.

14. DiRuggiero, J., F.T. Robb, R. Jagus, H.H. Klump, K.M. Borgest, M. Kessel, X. Mai, and M.W.W. Adams, "Characterization, cloning, and *in vitro* expression of the extremely thermostable glutamate dehydrogenase from the hyperthermophilic archaeon, ES4," J. Biol. Chem. 268:17767-17774, 1993.

15. Dong, G., C. Vieille, and J.G. Zeikus, "Cloning, sequencing and expression of the gene encoding the *Pyrococcus furiosus* amylopullulanase and enzyme characterization," Submitted to Appl. Environ. Microbiol., 1997.

16. Fiala, G., and K.O. Stetter, "*Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C," Arch. Microbiol. 145:56-61, 1986.

17. Fogarty, W.M., and C.T. Kelly, "Recent advances in microbial amylases," In Fogarty W.M., and C.T. Kelly (eds.), Microbial Enzymes and Biotechnology, 2nd edition, pp 71-121, Elsevier Applied Science, New York, 1990.

18. Fukusumi, S., A. Kamizono, S. Horinouchi, and T. Beppu, "Cloning and nucleotide sequence of a heat-stable amylase gene from an anaerobic thermophile, *Dictyoglomus thermophilum*," Eur. J. Biochem. 174:15-21, 1988.

19. Hain, J., W.-D. Reiter, U. Hudepohl, and W. Zillig, "Elements of an archaeal promoter defined by mutational analysis," Nucleic Acids Res. 20:5423-5428, 1992.

20. Henikoff, S., "Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing," Gene 28:351-359, 1984.

21. Horinouchi, S., S. Fukusumi, T. Ohshima, and T. Beppu, "Cloning and expression in *Escherichia coli* of two additional amylase genes of a strictly anaerobic thermophile, *Dictyoglomus thermophilum*, and their nucleotide sequences with extremely low guanine-plus-cytosine contents," Eur. J. Biochem. 176:243-253, 1988.

22. Jespersen, H.M., E.A. MacGregor, B. HenAssat, M.R. Sieks, and B. Svensson, "Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship of other amylolytic enzymes," J. Prot. Chem. 12:791-805, 1993.

23. Koch, R., P. Zablowski, A. Spreinat, and G. Antranikian, "Extremely thermostable amylolytic enzyme from archaebacterium *Pyrococcus furiosus*," FEMS Microbiol. Letters 71:21-26, 1990.

24. Koch, R., A. Sprehat, K. Lemke, and G. Antranikian, "Purification and properties of a hyperthermoactive α-Amylase from archaebacterium *Pyrococcus woesei*," Arch. Microbiol. 155:572-578, 1991.

25. Laderman, K.A., B.R. Davis, H.C. Krutzsch, M.S. Lewis, Y.V. Griko, P.L. Privalov, and C.B. Anfinsen, "The purification and characterization of an extremely thermostable α-Amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*," J. Biol. Chem. 268:24394-24401, 1993a.

26. Laderman, K.A., K. Asada, T. Uemori, H. Mukai, Y. Taguchi, I. Kato, and C.B. Anfinsen, α-Amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*, cloning and sequencing of the gene and expression in *Escherichia coli*," J. Biol. Chem. 268:24402-24407, 1993b.

27. Laemmli, U.K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," Nature 227:680-685, 1970.

28. Machius, M., G. Wiegand, and R. Huber, "Crystal structure of calcium-depleted *Bacillus licheniformis* α-Amylase at 2.2 Å resolution," J. Mol. Biol. 246:545-559, 1995.

29. McCrary, B.S., S.P. Edmondson, and J.W. Shriver, "Hyperthermophile protein folding thermodynamics: differential scanning calorimetry and chemical denaturation of Sac7d," J. Mol. Biol. 264:784-805,1996.

30. Perrin, D.D., and B. Dempsey (eds.), "Buffers for pH and Metal ion control," Chapman and Hall Ltd, London, 1979.

31. Qian, M., R. Haser., G. Buisson, E. Duée, and F. Payan, "The active center of a mammalian α-amylase. Structure of the complex of a pancreatic α-amylase with a carbohydrate inhibitor refined to 2.2 Å," Biochem. 33:6284-6294, 1994.

32. Qian, M., R. Haser, and F. Payan, "Structure and molecular model refinement of pig pancreatic α-amylase at 2.1 Å resolution," J. Mol. Biol. 231:785-799, 1993.

33. Reiter, W.-D., P. Palm, and W. Zillig, "Transcription termination in the archeabacterium *Sulfolobus*: signal structures and linkage to transcription initiation," Nucl. Acids Res. 16:2445-2459, 1988.

34. Robinson, K.A., and H.J. Schreier, "Isolation, sequence and characterization of the maltose-regulated *mlrA* gene from hyperthermophilic archaeum *Pyrococcus furiosus*," Gene 151:173-176, 1994.

35. Rüdiger, A., P.L. Jorgensen, and G. Antranikian, "Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*," Appl. Environ. Microbiol. 61:567-575, 1995.

36. Sanger, F., S. Nicklen, and A.R. Coulson, "DNA sequencing with chain terminating inhibitors," Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977.

37. Schumann, J., A. Wrba, R. Jaenicke, and K.O. Stetter, "Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*," FEBS Lett. 282:122-126, 1991.

38. Teplyakov, A.V., I.P. Kuranova, E.H. Harutyunyan, B.K. Vainshtein, C. Frömmel, W.E. Höhne, and K.S. Wilson, "Crystal structure of thermitase at 1.4Å resolution," J. Mol. Biol. 214:261-279, 1990.

39. Tiboni, O., P. Cammarano, and A.M. Sanangelantoni, "Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesei*: anomalous phylogenies inferred from analysis of archaeal and bacterial glutamine synthetase I sequences," J. Bacteriol. 175:2961-2969, 1993.

40. Tomazic, S.J., and A.M. Klibanov, "Mechanisms of irreversible thermal inactivation of *Bacillus* α-amylases," J. Biol. Chem. 263:3086-3091, 1988a.

41. Tomazic, S.J., and A.M. Klibanov, "Why is one *Bacillus* α-amylase more resistant against irreversible thermoinactivation than another"? J. Biol. Chem. 263:3092-3096, 1988b.

42. Vieille, C., D.S. Burdette, and J.G. Zeikus, "Thermozymes," Biotechnol. Annual Rev. 2:1-83, 1996.

43. Violet, M., and J.-C. Meunier, "Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase," Biochem. J. 263:665-670, 1989.

44. Volkin, D.B., and C.R. Middaugh, "The effect of temperature on protein structure," *In* Ahern, T.J., and M.C. Manning (eds.), Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation, Plenum Press, New York, 1992.

45. Voorhorst, W.G.B., R.I.L. Eggen, E.J. Luesink, and W.M. de Vos, "Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*," J. Bacteriol. 177:7105-7111, 1995.

46. Voorhorst, W.G.B., R.I.L. Eggen, A.C.M. Geerling, C. Platteeuw, R.J. Siezen, and W.M. de Vos, "Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*," J. Biol. Chem. 271:20426-20431, 1996.

47. Watson, M.E.E., "Compilation of published signal sequences," Nucleic Acids Res. 12:5145-5264, 1984.

48. Zwickl, P., S. Fabry, C. Bogedain, A. Haas, and R. Hensel, "Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*: characterization of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*," J. Bacteriol. 172:4329-4338, 1990.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Michigan Biotechnology Institute
(B) STREET: 3900 Collins Road
(C) CITY: Lansing
(D) STATE: Michigan
(E) COUNTRY: United States of America
(F) POSTAL CODE: 48909
(G) TELEPHONE: (517) 337-3181
(H) TELEFAX: (517) 337-2122

(ii) TITLE OF INVENTION: HYPERTHERMOSTABLE α-AMYLASE

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Quarles & Brady
(B) STREET: 411 East Wisconsin Avenue
(C) CITY: Milwaukee
(D) STATE: Wisconsin
(E) COUNTRY: U.S.A.
(F) ZIP: 53202-4497

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Baker, Jean C.
(B) REGISTRATION NUMBER: 35,433
(C) REFERENCE/DOCKET NUMBER: 660336.90641

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (414) 277-5709
(B) TELEFAX: (414) 271-3552

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 460 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Val Asn Ile Lys Lys Leu Thr Pro Leu Leu Thr Leu Leu Leu Phe Phe
1               5                  10                  15

Ile Val Leu Ala Ser Pro Val Ser Ala Ala Lys Tyr Leu Glu Leu Glu
            20                  25                  30

Glu Gly Gly Val Ile Met Gln Ala Phe Tyr Trp Asp Val Pro Gly Gly
        35                  40                  45

Gly Ile Trp Trp Asp His Ile Arg Ser Lys Ile Pro Glu Trp Tyr Glu
    50                  55                  60

Ala Gly Ile Ser Ala Ile Trp Leu Pro Pro Pro Ser Lys Gly Met Ser
65                  70                  75                  80
```

```
Gly Gly Tyr Ser Met Gly Tyr Asp Pro Tyr Asp Tyr Phe Asp Leu Gly
                85                  90                  95

Glu Tyr Tyr Gln Lys Gly Thr Val Glu Thr Arg Phe Gly Ser Lys Glu
            100                 105                 110

Glu Leu Val Arg Leu Ile Gln Thr Ala His Ala Tyr Gly Ile Lys Val
        115                 120                 125

Ile Ala Asp Val Val Ile Asn His Arg Ala Gly Gly Asp Leu Glu Trp
    130                 135                 140

Asn Pro Phe Val Gly Asp Tyr Thr Trp Thr Asp Phe Ser Lys Val Ala
145                 150                 155                 160

Ser Gly Lys Tyr Thr Ala Asn Tyr Leu Asp Phe His Pro Asn Glu Leu
                165                 170                 175

His Cys Cys Asp Glu Gly Thr Phe Gly Gly Phe Pro Asp Ile Cys His
            180                 185                 190

His Lys Glu Trp Asp Gln Tyr Trp Leu Trp Lys Ser Asn Glu Ser Tyr
        195                 200                 205

Ala Ala Tyr Leu Arg Ser Ile Gly Phe Asp Gly Trp Arg Phe Asp Tyr
    210                 215                 220

Val Lys Gly Tyr Gly Ala Trp Val Val Arg Asp Trp Leu Asn Trp Trp
225                 230                 235                 240

Gly Gly Trp Ala Val Gly Glu Tyr Trp Asp Thr Asn Val Asp Ala Leu
                245                 250                 255

Leu Ser Trp Ala Tyr Glu Ser Gly Ala Lys Val Phe Asp Phe Pro Leu
            260                 265                 270

Tyr Tyr Lys Met Asp Glu Ala Phe Asp Asn Asn Asn Ile Pro Ala Leu
        275                 280                 285

Val Tyr Ala Leu Gln Asn Gly Gln Thr Val Val Ser Arg Asp Pro Phe
    290                 295                 300

Lys Ala Val Thr Phe Val Ala Asn His Asp Thr Asp Ile Ile Trp Asn
305                 310                 315                 320

Lys Thr Pro Ala Tyr Ala Phe Ile Leu Thr Tyr Glu Gly Gln Pro Val
                325                 330                 335

Ile Phe Tyr Arg Asp Phe Glu Glu Trp Leu Asn Lys Asp Lys Leu Ile
            340                 345                 350

Asn Leu Ile Trp Ile His Asp His Leu Ala Gly Gly Ser Thr Thr Ile
        355                 360                 365

Val Tyr Tyr Asp Asn Asp Glu Leu Ile Phe Val Arg Asn Gly Asp Ser
    370                 375                 380

Arg Arg Pro Gly Leu Ile Thr Tyr Ile Asn Leu Ser Pro Asn Trp Val
385                 390                 395                 400

Gly Arg Trp Val Tyr Val Pro Lys Phe Ala Gly Ala Cys Ile His Glu
                405                 410                 415

Tyr Thr Gly Asn Leu Gly Gly Trp Val Asp Lys Arg Val Asp Ser Ser
            420                 425                 430

Gly Trp Val Tyr Leu Glu Ala Pro Pro His Asp Pro Ala Asn Gly Tyr
        435                 440                 445

Tyr Gly Tyr Ser Val Trp Ser Tyr Cys Gly Val Gly
    450                 455                 460
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1542 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTATTAGAT TTTGACGTGC GTTGATGAAC ATTTATGTTC ACATGATCAT AACAGAAAAT 60

TTATATGTAT CATCACCAGT GATACATTAT GAGACTTTGG TGTATGGAGG TGATCACGTG 120

AACATAAAGA AATTAACACC CCTCCTAACT CTATTACTGT TTTTTATAGT ACTAGCAAGT 180

CCAGTAAGTG CAGCAAAATA CTTGGAGCTT GAAGAGGGAG GAGTTATAAT GCAAGCATTC 240

TATTGGGATG TTCCAGGGGG AGGAATTTGG TGGGATCATA TAAGATCGAA GATTCCTGAA 300

TGGTATGAAG CTGGAATCTC TGCAATATGG CTACCTCCAC CAAGCAAGGG GATGAGTGGA 360

GGATATTCAA TGGGCTACGA TCCCTATGAT TACTTTGATC TCGGCGAGTA CTACCAGAAG 420

GGAACTGTAG AGACGCGTTT TGGATCAAAA GAAGAACTAG TGAGATTGAT ACAAACTGCC 480

CATGCCTATG GAATAAAGGT AATCGCCGAT GTAGTTATAA ACCACAGGGC TGGTGGTGAC 540

CTAGAATGGA ACCCCTTCGT TGGAGATTAC ACATGGACAG ACTTTTCTAA AGTTGCCTCA 600

GGGAAATATA CAGCTAACTA TCTGGACTTC CATCCAAACG AGCTTCATTG TTGTGACGAA 660

GGAACCTTTG GAGGATTTCC AGATATATGT CATCACAAAG AGTGGGATCA GTACTGGCTA 720

TGGAAGAGCA ATGAGAGTTA TGCTGCTTAT TTAAGAAGCA TAGGATTTGA TGGTTGGAGA 780

TTTGACTATG TTAAGGGCTA TGGAGCTTGG GTTGTCAGAG ACTGGCTTAA TTGGTGGGGA 840

GGTTGGGCAG TTGGAGAGTA CTGGGACACA AATGTAGATG CACTACTAAG CTGGGCATAT 900

GAGAGTGGTG CAAAGGTCTT TGACTTCCCG CTCTACTATA AAATGGATGA AGCATTTGAC 960

AATAACAACA TTCCAGCATT AGTCTATGCC CTACAAAACG GACAAACTGT AGTTTCGAGA 1020

GATCCATTTA AGGCAGTAAC TTTCGTTGCC AATCATGACA CAGATATAAT ATGGAACAAG 1080

TATCCAGCAT ATGCGTTCAT ATTGACATAT GAGGGACAGC CAGTAATATT CTACAGGGAC 1140

TTTGAGGAAT GGCTGAACAA GGATAAGCTA ATTAACCTCA TTTGGATCCA TGATCATTTG 1200

GCAGGAGGAA GCACAACAAT TGTCTACTAC GACAACGATG AGCTCATATT TGTGAGAAAT 1260

GGAGATTCTA GAAGGCCTGG GCTTATAACT TACATTAACT TGAGCCCTAA CTGGGTTGGT 1320

AGGTGGGTAT ACGTTCCAAA GTTTGCAGGG GCTTGTATTC ATGAATACAC TGGAAACCTA 1380

GGAGGATGGG TAGATAAAAG AGTAGATAGT AGCGGATGGG TATACCTAGA GGCACCACCT 1440

CACGATCCAG CTAACGGCTA CTATGGGTAC TCCGTATGGA GTTATTGTGG TGTTGGGTGA 1500

CTTTTTCTTT TTTCTTTTTA ACAATGGGAG AAGTGCAAAT AC 1542

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 510 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1               5                  10                  15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
            20                  25                  30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
        35                  40                  45

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
    50                  55                  60

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65                  70                  75                  80

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
                85                  90                  95

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
            100                 105                 110

Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly Asp
        115                 120                 125

Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val Thr
    130                 135                 140

Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly Glu
145                 150                 155                 160

His Arg Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly Ser
                165                 170                 175

Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr Asp
            180                 185                 190

Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly Lys
        195                 200                 205

Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr Leu
    210                 215                 220

Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu Ile
225                 230                 235                 240

Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly Phe
                245                 250                 255

Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp Trp
            260                 265                 270

Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val Ala
        275                 280                 285

Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys
    290                 295                 300

Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln Phe
305                 310                 315                 320

His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu Leu
                325                 330                 335

Asn Ser Thr Val Val Ser Lys His Pro Leu Lys Ala Val Thr Phe Val
            340                 345                 350

Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val Gln
        355                 360                 365
```

Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser
370 375 380

Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp
385 390 395 400

Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile Leu
405 410 415

Lys Ala Arg Lys Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp His
420 425 430

His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val Ala Asn
435 440 445

Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala Lys Arg
450 455 460

Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp Ile Thr
465 470 475 480

Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp Gly Glu
485 490 495

Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
500 505 510

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAAATGTCAC GTTGTATGG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGAGTGGTG CAAAGGTC 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGGTGGTGA CCTAGAATG 19

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TATCTGTGTC ATGATTGGC 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGGTGATCA CATGAACATA AAGAAATIAA CACC 33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGTTAATT TCTTTATGTT CATGTGATCA CCT 33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCTAGCTTG GAGCTTGAAG AGGGAG 26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTCGAGACC ACAATAACTC CATACGGAG 29

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTGGG 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCGAG 6

CLAIMS

We claim:

1. A preparation of *Pyrococcus furiosus* extracellular α-amylase.

2. The preparation of claim 1, wherein the α-amylase has an optimal temperature of 100°C.

3. The preparation of claim 1, wherein the α-amylase has a pH optimum of 5.5-6.0.

4. The preparation of claim 1, wherein the α-amylase has a half-life at 98°C of between 12-14 hours.

5. The enzyme of claim 1, wherein the amino acid sequence of the enzyme comprises SEQ ID NO:1.

6. A preparation of α-amylase enzyme, wherein the enzyme has an optimal temperature of 100°C.

7. The enzyme of claim 6 wherein the enzyme has a pH optimum of 5.5-6.0.

8. The enzyme of claim 6, wherein the enzyme has a half-life at 98°C of 12-14 hours.

9. The enzyme of claim 6, wherein the enzyme has a molecular weight of 95,000-105,000, as measured by gel electrophoresis.

10. A method of hydrolyzing starch comprising the step of mixing starch and the α-Amylase of claim 1 under conditions suitable for enzyme activity, wherein the starch is degraded into $G_2$-$G_7$ oligosaccharides.

11. A method of hydrolyzing starch comprising the step of mixing starch and the α-Amylase of claim 6 under conditions suitable for enzyme activity, wherein the starch is degraded into $G_2$-$G_7$ oligosaccharides.

12. A method of creating an α-amylase preparation comprising

(a) obtaining a nucleotide sequence encoding an α-amylase, wherein the encoded enzyme has an optimal temperature of 100°C, and

(b) placing the gene sequence into an expression vector, so that the sequence is expressed as a mature protein.

13. The method of claim 12 wherein the gene sequence is obtained from *Pyrococcus furiosus*.

14. The method of claim 13 wherein the gene sequence comprises residues 118 through 1497 of SEQ ID NO:2.

15. An isolated DNA sequence encoding an extracellular α-amylase, wherein the α-amylase has an optimal temperature of 100°C.

16. The sequence of claim 15 wherein the sequence is obtained from *Pyrococcus furiosus*.

17. The sequence of claim 16, wherein the sequence comprises SEQ ID NO:2.

18. The sequence of claim 16, wherein the sequence comprises residues 118 through 1497 of SEQ ID NO:2.

```
1
TTTATTAGATTTTGACGTGCGTTGATGAACATTTATGTTCACATGATCATAACAGAAAAT
                                      -35
Box A                                                  RBS
TTATATGTATCATCACCAGTGATACATTATGAGACTTTGGTGTATGGAGGTGATCACGTG
-10/-35                  -10                                V
121
AACATAAAGAAATTAACACCCCTCCTAACTCTATTACTGTTTTTTATAGTACTAGCAAGT
N  I  K  K  L  T  P  L  L  T  L  L  L  F  F  I  V  L  A  S
181
CCAGTAAGTGCAGCAAAATACTTGGAGCTTGAAGAGGGAGGAGTTATAATGCAAGCATTC
P  V  S  A  A  K  Y  L  E  L  E  E  G  G  V  I  M  Q  A  F
241           *  *  *  *  *
TATTGGGATGTTCCAGGGGGAGGAATTTGGTGGGATCATATAAGATCGAAGATTCCTGAA
Y  W  D  V  P  G  G  G  I  W  W  D  H  I  R  S  K  I  P  E
301
TGGTATGAAGCTGGAATCTCTGCAATATGGCTACCTCCACCAAGCAAGGGGATGAGTGGA
W  Y  E  A  G  I  S  A  I  W  L  P  P  P  S  K  G  M  S  G
361
GGATATTCAATGGGCTACGATCCCTATGATTACTTTGATCTCGGCGAGTACTACCAGAAG
G  Y  S  M  G  Y  D  P  Y  D  Y  F  D  L  G  E  Y  Y  Q  K
421
GGAACTGTAGAGACGCGTTTTGGATCAAAAGAAGAACTAGTGAGATTGATACAAACTGCC
G  T  V  E  T  R  F  G  S  K  E  E  L  V  R  L  I  Q  T  A
481
CATGCCTATGGAATAAAGGTAATCGCCGATGTAGTTATAAACCACAGGGCTGGTGGTGAC
H  A  Y  G  I  K  V  I  A  [D  V  V  I  N  H]  R  A  G  G  D
541                          I
CTAGAATGGAACCCCTTCGTTGGAGATTACACATGGACAGACTTTTCTAAAGTTGCCTCA
L  E  W  N  P  F  V  G  D  Y  T  W  T  D  F  S  K  V  A  S
601
GGGAAATATACAGCTAACTATCTGGACTTCCATCCAAACGAGCTTCATTGTTGTGACGAA
G  K  Y  T  A  N  Y  L  D  F  H  P  N  E  L  H  C  C  D  E
661
GGAACCTTTGGAGGATTTCCAGATATATGTCATCACAAAGAGTGGGATCAGTACTGGCTA
G  T  F  G  G  F  P  D  I  C  H  H  K  E  W  D  Q  Y  W  L
721
TGGAAGAGCAATGAGAGTTATGCTGCTTATTTAAGAAGCATAGGATTTGATGGTTGGAGA
W  K  S  N  E  S  Y  A  A  Y  L  R  S  I  G  F  D  [G  W  R]
781
TTTGACTATGTTAAGGGCTATGGAGCTTGGGTTGTCAGAGACTGGCTTAATTGGTGGGGA
[F  D  Y  V  K  G]  Y  G  A  W  V  V  R  D  W  L  N  W  W  G
841  II
GGTTGGGCAGTTGGAGAGTACTGGGACACAAATGTAGATGCACTACTAAGCTGGGCATAT
G  W  A  V  G  [E  Y  W  D]  T  N  V  D  A  L  L  S  W  A  Y
901              III
GAGAGTGGTGCAAAGGTCTTTGACTTCCCGCTCTACTATAAAATGGATGAAGCATTTGAC
E  S  G  A  K  V  F  D  F  P  L  Y  Y  K  M  D  E  A  F  D
961
AATAACAACATTCCAGCATTAGTCTATGCCCTACAAAACGGACAAACTGTAGTTTCGAGA
N  N  N  I  P  A  L  V  Y  A  L  Q  N  G  Q  T  V  V  S  R
1021
GATCCATTTAAGGCAGTAACTTTCGTTGCCAATCATGACACAGATATAATATGGAACAAG
D  P  F  K  A  V  T  [F  V  A  N  H  D]  T  D  I  I  W  N  K
1081                        IV
TATCCAGCATATGCGTTCATATTGACATATGAGGGACAGCCAGTAATATTCTACAGGGAC
Y  P  A  Y  A  F  I  L  T  Y  E  G  Q  P  V  I  F  Y  R  D
1141
TTTGAGGAATGGCTGAACAAGGATAAGCTAATTAACCTCATTTGGATCCATGATCATTTG
F  E  E  W  L  N  K  D  K  L  I  N  L  I  W  I  H  D  H  L
1201
GCAGGAGGAAGCACAACAATTGTCTACTACGACAACGATGAGCTCATATTTGTGAGAAAT
A  G  G  S  T  T  I  V  Y  Y  D  N  D  E  L  I  F  V  R  N
1261
GGAGATTCTAGAAGGCCTGGGCTTATAACTTACATTAACTTGAGCCCTAACTGGGTTGGT
G  D  S  R  R  P  G  L  I  T  Y  I  N  L  S  P  N  W  V  G
1321
AGGTGGGTATACGTTCCAAAGTTTGCAGGGGCTTGTATTCATGAATACACTGGAAACCTA
R  W  V  Y  Y  V  P  K  F  A  G  A  C  I  H  E  Y  T  G  N  L
1381
GGAGGATGGGTAGATAAAAGAGTAGATAGTAGCGGATGGGTATACCTAGAGGCACCACCT
G  G  W  V  D  K  R  V  D  S  S  G  W  V  Y  L  E  A  P  P
1441
CACGATCCAGCTAACGGCTACTATGGGTACTCCGTATGGAGTTATTGTGGTGTTGGGTGA
H  D  P  A  N  G  Y  Y  G  Y  S  V  W  S  Y  C  G  V  G  ***
1501
CTTTTTCTTTTTTCTTTTTAACAATGGGAGAAGTGCAAATAC
```

FIG.1

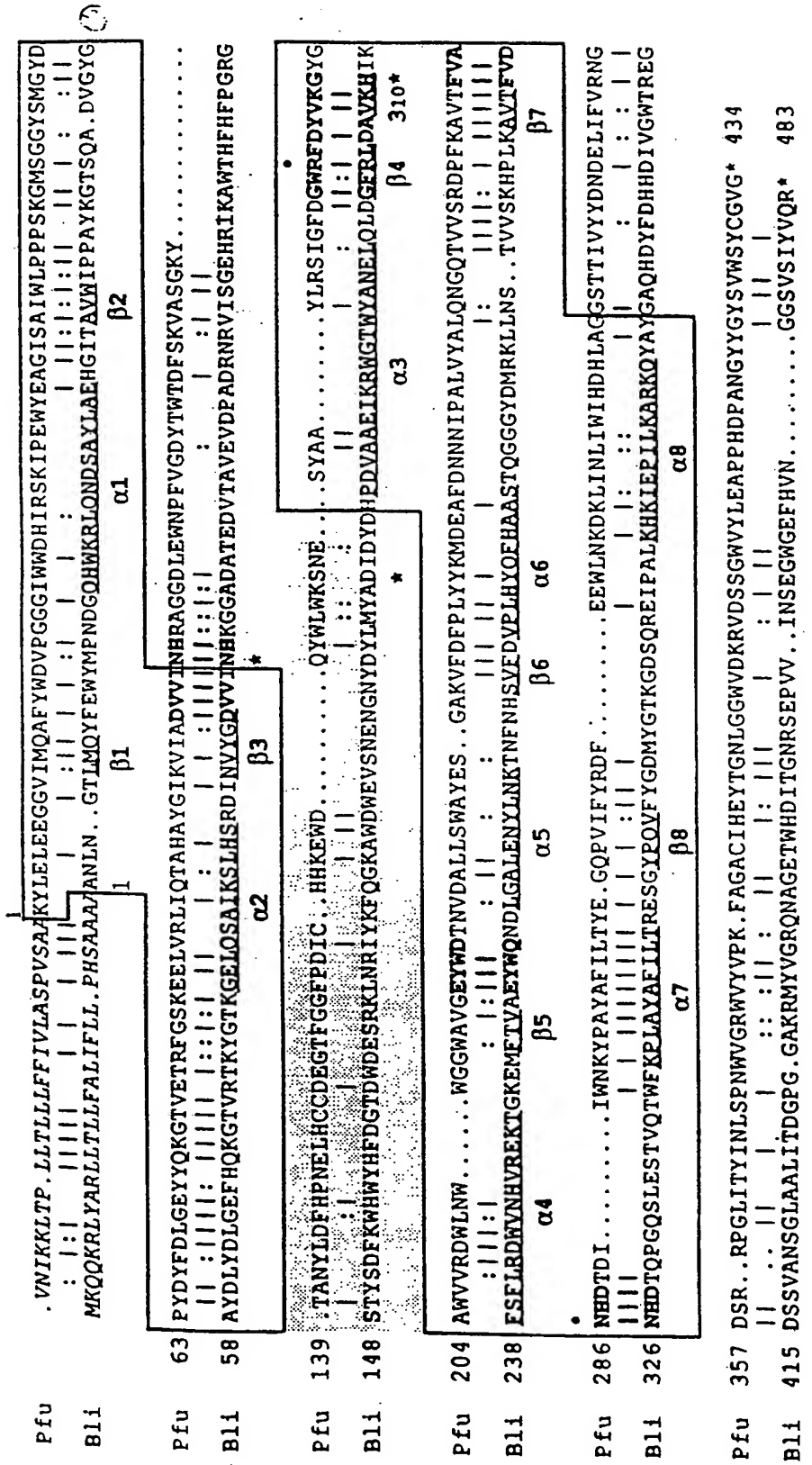

FIG. 2

FIG. 3A

FIG. 3B

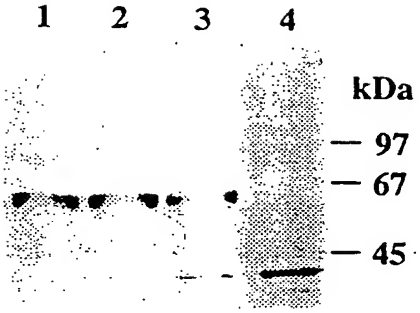


FIG. 3C

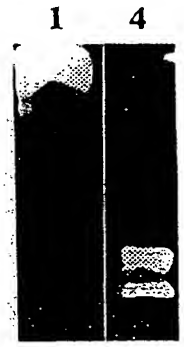


FIG. 4A

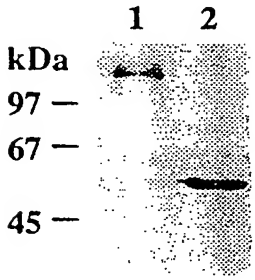


FIG. 4B

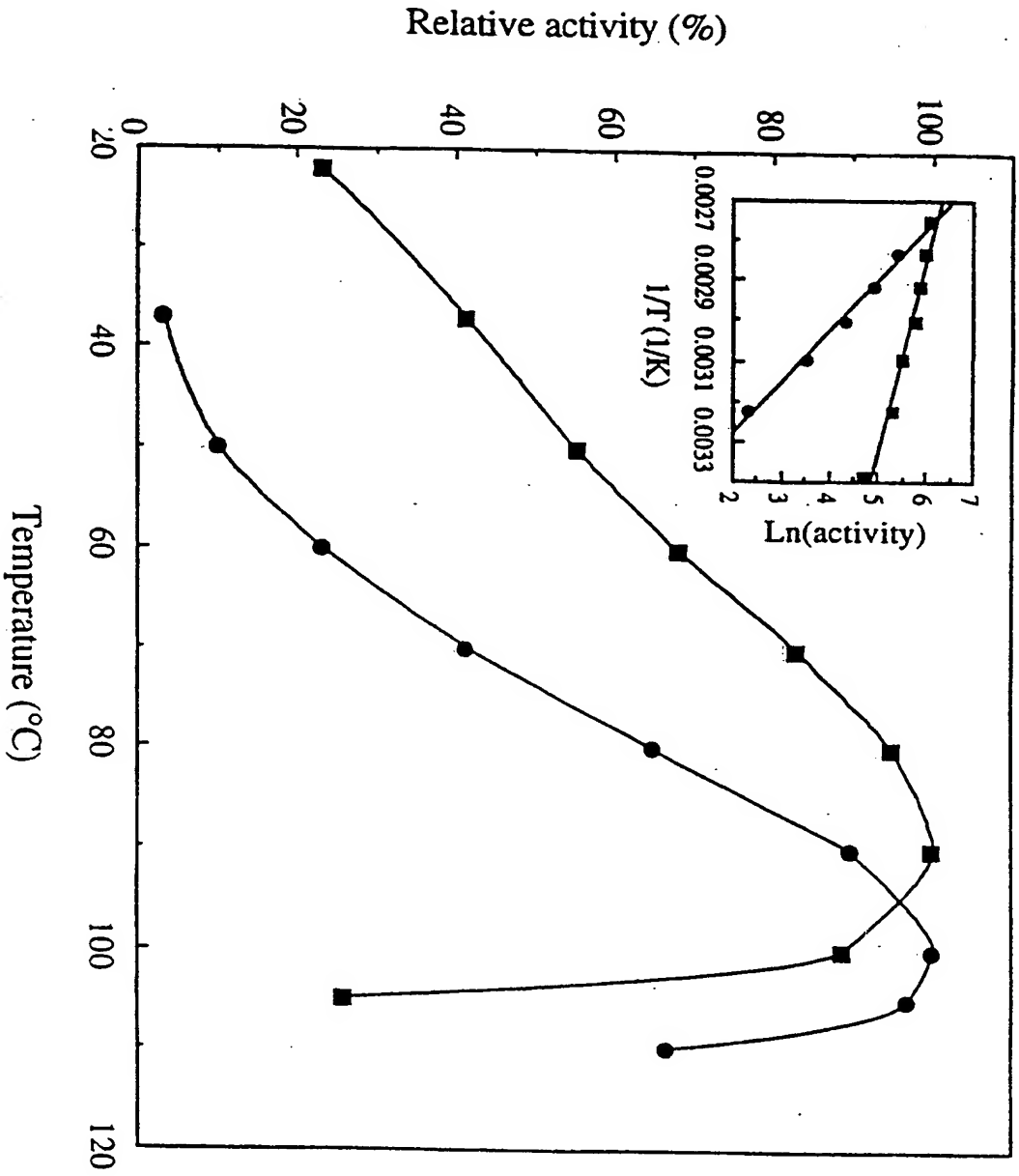


FIG. 5

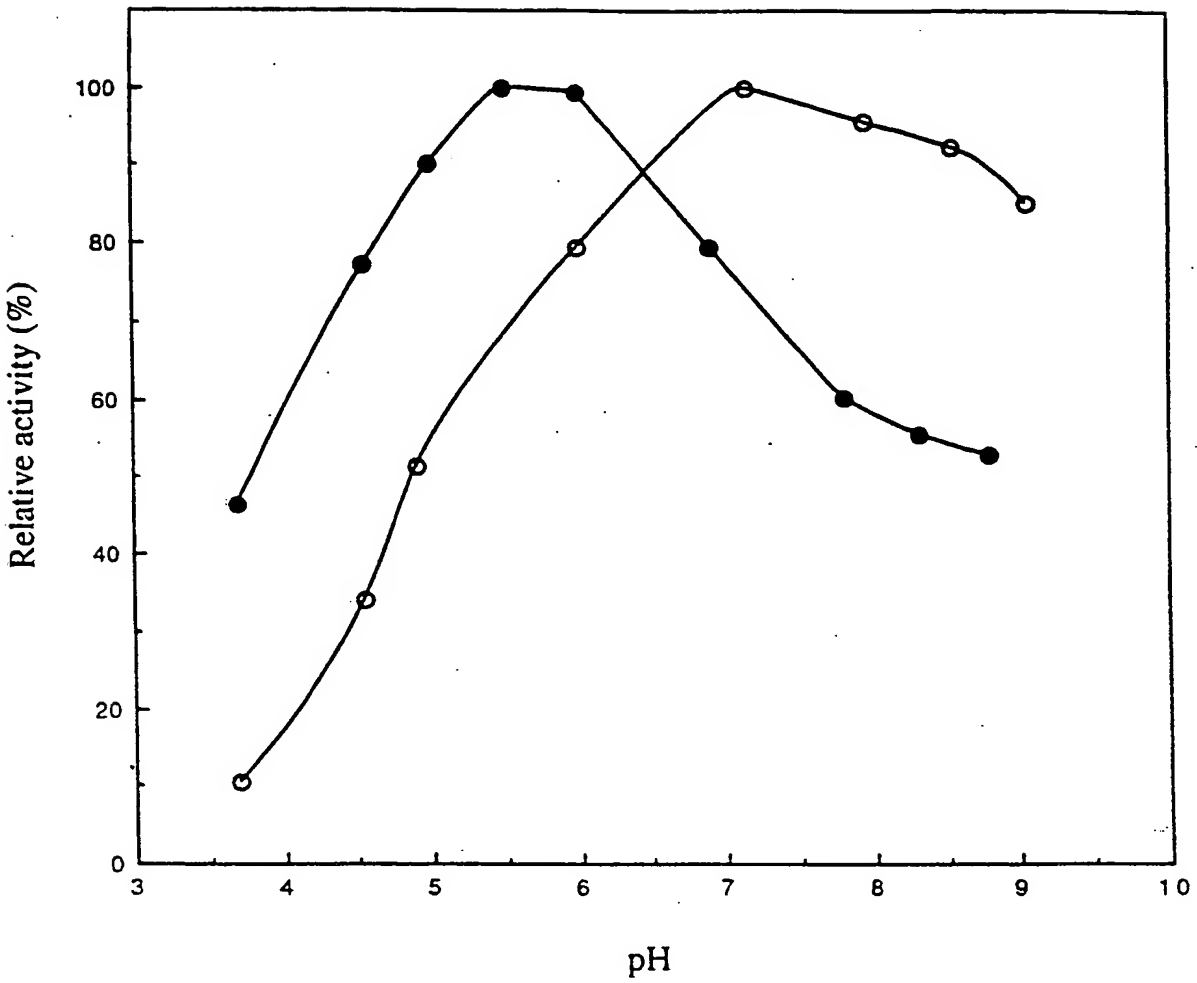


FIG. 6

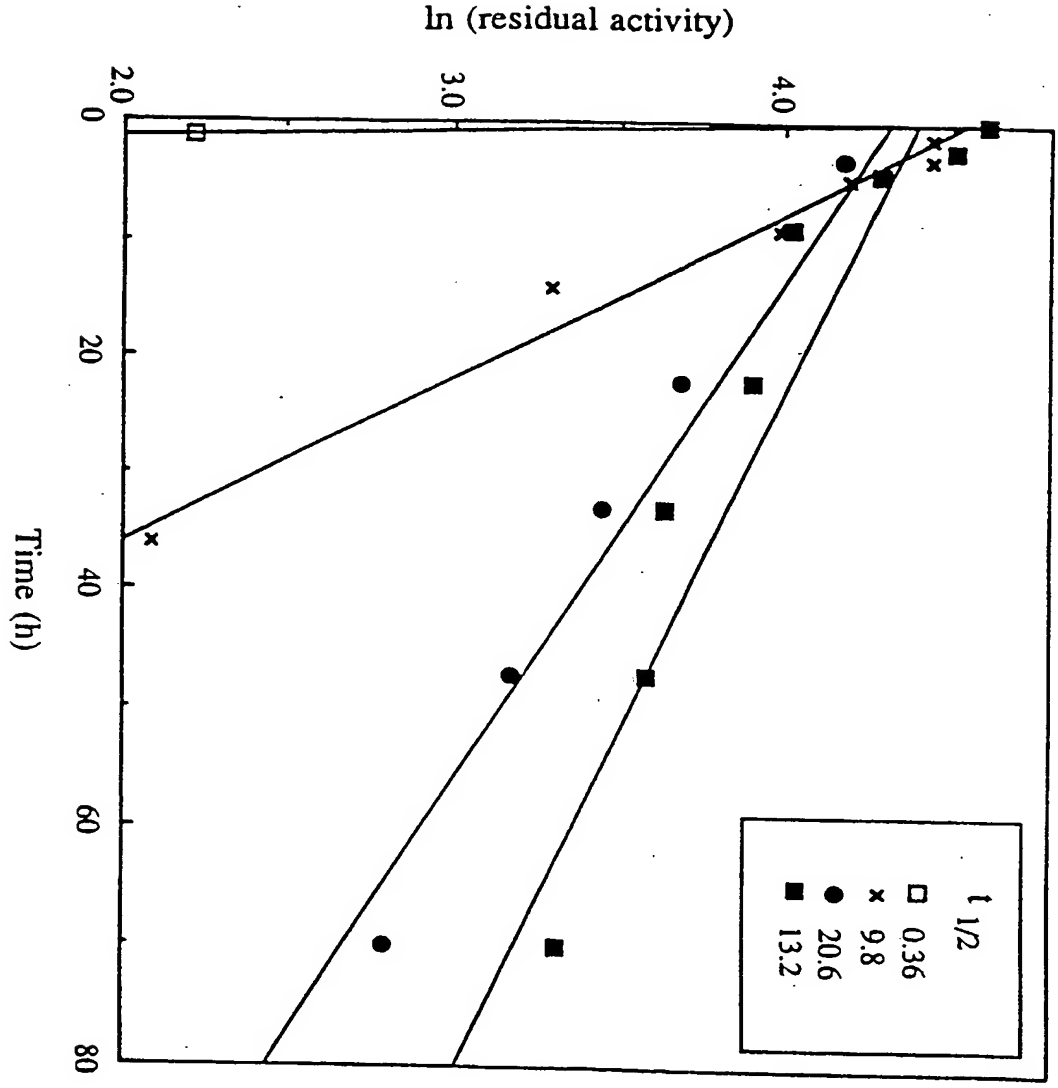


FIG. 7

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07192

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12N 9/28, 15/56
US CL :435/202; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/202; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: alpha-amylase, Pyrococcus furiousus, extracellular, secret?, thermostable

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | KOCH, et al. Extremely thermostable amylolytic enzyme from the archaebacterium Pyrococcus furiosus. FEMS Microbiology Letters. 1990. Vol. 71. pages 21-26, see entire document. | 1-11<br>-------<br>12-18 |
| X<br>---<br>Y | LADERMAN et al. The Purification and Characterization of an Extremely Thermostable alpha-Amylase from the Hyperthermophilic Archaebacterium Pyrococcus furiosus. The Journal of Biological Chemistry. 15 November, 1993. Vol. 268. No. 32. pages 24394-24401, see entire document. | 1-11<br>------<br>12-18 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "B" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>25 JUNE 1998 | Date of mailing of the international search report<br>11 AUG 1998 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>KEITH D. HENDRICKS<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07192

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | LADERMAN et al. Alpha-Amylase from the Hyperthermophilic Archaebacterium Pyrococcus furiousus. The Journal of Biological Chemistry. 15 November 1993. Vol. 268. No. 32. pages 24402-24407, see entire document. | 1-18 |